A synopsis entitled

“ In vitro regeneration studies for salt tolerance in Safflower”

Swami Ramananda Teerth Marathwada University, Nanded

For Registration of Ph.D.

In

Biotechnology

स्वामी रामानंद तीर्थ मराठवाडा विद्यापीठ, नांदेड.

Under the Faculty of Science

: Submitted By :

Wagh Hemant Anilrao

Research Scholer

Research Centre in Biotechnology,

Swami Vivekanand College, Udgir

Under the Guidance of

Dr. B.M.Kareppa

Head Department of Biotechnology

DSM College, Parbhani

ABSTRACT OF PROPOSED WORK PLAN-

Abiotic stress factors are the main limitation to plant growth and yield in agriculture. Among them, salinity stress caused by accumulation of salt in soil, is probably the most impacting adverse condition and the most widely encountered by plants, not only in crop fields but also in wild environments. Abiotic stress leads to a series of morphological, physiological, biochemical, and molecular changes that adversely affect plant growth and productivity . Drought, salinity, extreme temperatures, and oxidative stress are often interconnected, and may induce similar cellular damage. Many studies have reported that the *in vitro* culture alone or combined with mutagenesis, induced with physicochemical or biological agents, can be exploited to increase genetic variability and mutants, as a potential source of new commercial cultivars. *In vitro* culture is the efficient method to develop genotype having capacity to resist against salinity. *In vitro* culture environments can be mutagenic and plants regenerated from organ cultures, calli, protoplasts and via somatic embryogenesis sometimes exhibit phenotypic and/or genotypic variations. It is important to point that tissue culture increases the efficiency of mutagenic treatments and allows handling of large populations and rapid cloning of selected variants. The similarities of the effects induced by the stress in the plant cultured *in vitro* and *in vivo* conditions suggest that the *in vitro* system can be used as an alternative to field evaluations for studying the general effect of water-stress On plantgrowth and development. So, present study will be carried out to standardize protocol of in vitro regeneration of calli and to screen the genotype against salinity.

INTRODUCTION :

Safflower belongs to the Division Phanerogram, Sub. Division Angiosperm, Tribe Tubifloreae. Family Compositae and Sub family Asteraceae. There are 36 species in this genus. Out of which *Carlhamus tinctorius L.* (2n=24) is the only cultivated species of safflower which is used for oil production and the rest are wild species. Safflower *(Carlhamus tinctorius* L.) owes its importance to the demand for oil, which results from the highly polyunsaturated/saturated ratios of fatty acids coupled with elevated levels-of a- tocopherol (Sujatha, 2002) as they help in educing the cholesterol level in blood (Singh and Nimbkar 2006).The safflower oil has good drying property and therefore used in manufacturing paints, warnishes and linoleum. Recently safflower petals have been used for their good medicinal and cosmetic value. Petals prepared tea i.e. herbal tea removes constipation (Boland, 2006). In colouring textiles, safflower's dried

flowers are used as a natural textile dye. Natural dyes derived from plants are not widely used in industry but it is getting more important world wide because of naturality jashion trends. The colourful matter in safflower is benzoquinone-based Carthamin, so it is one of the quinone type natural dyes. It is a direct dye (Cl Natural Red 26 and soluble. The latter is also used in painting in the place of linseed oil, particularly with white, as it does not have the yellow tint which linseed oil possesses. Moreover, safflower is reported to be the best example of crop with variability for fatty acid composition in seed-oil (Knowles, 1989).

In vitro selection technique has txc.i used to improve abiotic environmental stresses such as cold hardiness. salt tolerance and drought tolerance (Zair *et al.,* 2003; Bajji *el al.,* 2004;Gawande et al. 2005). Recent progress in the genetic manipulation of plant cells through tissue culture has opened new possibilities in crop improvement in which crop species can be improved without interfering with a large portion of the genes and without introducing alien or exogenous genetic material (Farshadlar *ci al.,* 2012). Drought and salinity reduces the ability of plant to take up water and decrease growth rate, photosynthesis rate and stomatal conductance of plants (Shabani *el cd.* 2013). Drought and salinity are becoming widespread regions, and may cause serious salinisation of more than 50% of all arable land by 2050 (Wang *et al.,* 2003). Improved yields of crop depend on many factors, among which one-of the most important is tolerance to environmental stress, particularly to water stress indeed in Safflower, drought is a major non-biotic stress that causes severe yield loss. Many researchers have suggested that selection is more convenient and practicable if the plant species possesses distinctive indicators of salt tolerance at the whole plant, tissue or .crlinded level (Ashraf and Harris, 2004, Munns, 2002).

1. REV1EW OF LITERATURE AND DEVELOPMENT IN THE SUBJECT-

Maxdal (2001) induced adventitious shoot buds directly on the adaxial surface of the Cotelydons of eight safflower cultivars after 14 d of culture initiation on Murashige and Skoog's (MS) medium supplemented with various levels of 6- benzylaminopurine (MA). Maximum shoot organogenesis of 54.4% with 10.2 shoots per responding cotyledon was 3 obtained with 8.87 mM BA in the cv. S-144.

Munns (2002) showed that salinity reduces the ability of plants to take up water, which quickly reduces the growth rate, as well as a number of metabolic changes identical to those Caused by water stress.

Wang (2005) observed that *Carthamus* L. genus consists of about 25 main species in the world. Species C. *tinctorius* (safflower) corolla is known as an important crude drug in traditional Chinese medicine for promoting blood circulation and removing blood stasis. It has been used clinically in the Precention and treatment of cardio-vascular and thrombotic diseases.

Dics (2006) studied that the productivity of several commercial crops is limited by major abiotic stresses including salinity, drought, water logging, heat, frost and mineral toxicity Successful application of biotechnology to abiotic constraints being faced by the Scoops will require knowledge of good biological information regarding the target species as well as the mechanisms underlying resistance/tolerance to these stresses.

Konar et *al.* (2006) established efficient somatic embryogenesis in *Carthamus tinctorius* (t.) in Which primary and secondary 'embryogenic calluses were developed from hypocotyls and primay cotyledonary somatic embryos (PCSEs) having two types of callus. At the biochemical level, two somatic embryogenesis systems were different. Both primary and secondary adventives somatic embryogenesis and the role of plant growth regulators in two modes of somatic embryo formation have been discussed.

3. OBJECTIVES OF RESEARCH

1. Development *of in-vitro* regeneration protocol in elite safflower genotype
2. *In-vitro* screening of calli for selection against salinity tolerance
3. Physico-Biochemical studies of stressed tolerant calli

4. METHODOLOGY TO BE ADOPTED-

Materials

Genotype : AKS 207

Explant:

- Hypocotyl

A) Regeneration of explants :

- **Preparation of Explants**

- **Induction of callus : 2,4-D, kinetin, BAP and NAA**

1. **Hypocotyls explants on MS+NAA**

1 .00mg/lit
1.50mg/lit
2.00mg/lit
2.50mg/lit

2. **Hypocotyls explants on MS+BAP**

1 .00mg/lit
1.50mg/lit
2.00mg/lit
2.50mg/lit

3. **Hypocotyls explants on MS+2,4-D**

1.00mg/lit
l.50mg/lit
2.00mg/lit
2.50ing/l it

4. **Hypocotyls explants on MS-r2,4-D+Kinetin**

1.00mg/lit
1.50mg/lit
2.00mg/lit
2.50mg/lit

B) Shoot Regeneration from established callus:

- Shoot formation : BAP, NAA and combination with TDZ

1. **MS+BAP**
   MS+ 1,50mg/lit
   MS.+ 2.00mg/lit
   MS+ 2.50mg/lit
   MS+ 3.00mg/lit

2. **MS+NAA**
   MS' l.50mg/lit
   MS+ 2.00mg/lit

MS+ 2.50mg/lit
MS+ 3.00mg/li

3. **MS+TDZ+(NAA or BAP)**

MS+0.50mg/lit+0.50mg/lit
MS+0.50mg/lit+l.00mg/lit
MS+0.50mg/lit+1.50mg/lit
MS+1.00mg/lit+0.50mg/lit
MS+1.00mg/lit+1.00mg/lit
MS+1.00mg/lit+1.50mg/lit
MS+1.50mg/lit+0.50mg/lit
MS+1,50mg/lit+l.00mg/lit
MS+1 50mgZlit+1.50mg/lit
MS+2.00mg/lit+0.50mg/lit
MS+2.00mg/iit+1 .00nig/lit
MS+2.00mg/lit+1.50mg/lit

Shoot regeneration from established shooting :

- Shoot multiplication : BAP,NAA and **combination with TDZ**

MS+0.50mg/lit+0.50mg/lit

MS+0.50mg/lit+1 .00mg/lit

MS+0.50mg/lit+ l .50mg/lit

MS+1.00mg/lit+0.50mg/lit

MS+1.00mg/lit+ l. 00mg/lit

MS+1.00mg/lit+1.50mg/lit

MS+1,50mg/lit+0.50mg/lit

MS+1.50mg/lit+1.00mg/lit

MS+1.50mg/lit+l .50mg/lit

MS+2.00mg/lit+0.50mg/lit

MS+2.00mg/lit+l. 00mg/lit

MS+2.00mg/lit+l ,50mg/lit

C) In vitro selection of calli for salinity tolerance:

- NaCI

Nacl concentration 0,50,100,200,300,400mM

D) Physiological and biochemical analysis of stressed Calli

- Chlorophyll content
- Total Protein
- Proline
- Lipid peroxidation (MDA)
- SuperOxi.de dismutase
- Peroxidase
- Catalase

5. IMPORTANCE OF STUDY

National and International status

Lupi *el al.*. (2004) described a method for callus induction, adventitious bud regeneration, shoot multiplication and rooting of in vitro formed shoots of *Melianthus annuus* L. var. Argentario is described. Hypocotyl and cotyledon explants formed callus on medium containing 2 mgl^{+4} raplitialene acetic acid and 0.5 $mgl"^{1}$ benzyladenine. Adventitious buds were formed on hypocotyl Segments on medium containing 0.5-2 mgl^{+4} benzyladenine. The optimal level of sucrose concentration for shoot regeneration from hypocotyls was 1.5%. Multiplication from shoot apices was promoted by kinetin (2 mgl^{+1}) plus gibberellic acid (5 mgl^{-1}), benzyladenine (2 mgl^{+1}) plus gibberellic acid(10 mgl^{+1}') or at lower frequency by benzyladenine (1 mgl^{+1})

Sujatha and Dinesh (2007) observed highly prolific adventitious shoot regeneration was in C *xasicMrius* and *C. arborescent* on both growth regulator combinations and the shoot regeneration frequency was higher on medium supplemented with TDZ + NAA. Nodal culture of nine carthamus species on media supplemented with BA and kinetin (KIN) individually revealed the Superiority of media supplemented with BA over that of KIN in facilitating a higher shoot proliferation index.- Proliferating shoots- from axillary buds and leaf explants-were transferred to medium supplemented with 1.0 mg dm^{-3} KIN or 0.5 mg dm^{-3} BA for shoot elongation. Elongated shoots were rooted on half-strength MS medium supplemented with 1.0 mg $dm"^{3}$ each of indole- buteryic (IBA) and phloroglucinol.

Narsimha Rao N *el.al* (2008) was obtained best regeneration response on the medium Supplemented with TDZ 1 .Omg/1 + NAA 0.2mg/l. Shoots emerged as clusters within 2 weeks of culture from all the explants. Root explants gave shoots on medium containing TDZ 1.0mg/l

+ NAA 0.2 mg/l while the hypocotyls gave regeneration with *TDZ* l.Omg/1 + NAA 0.2 mg/l. Shoots regenerated from explants were first transferred to 0-5mg/l or 1.Omg/1 kinetin and later transferred to 0.2mg/l BA medium for shoot elongation. For inducing rooting the elongated shoots were transferred to different rooting media. Best rooting was Obtained on MS medium supplemented with phloroglucinol atlmg/1 and IBA 0.2mg/l Concentration Kill curve studies indicated that hygromycin at 15 mg/l and phosphinothricin mg/l could act as effective selection agents for identifying the transformed shoots.

Zurich Soheilikhah *el.al* (2013) suggested that the physiological mechanisms which mediate the response to salt and drought stress are different. It is noteworthy that growth retardation and reduced cell viability were .associated with a loss of turgor under mannitol-induced stress and conspicuous increase in Na+ but a corresponding decline in K+ concentrations, demonstrating a typical glycophytic nature of safflower. Moreover, ion status in safflower cells is closely related to the nature of the stress factor applied in the medium. The accumulation of salt ions (Na+ and K+), osmolytes (proline) and retention of an adequate water status may have an important role in osmotic adjustment in safflower genotypes under Mannitol stress.

M. Kakari *e/.al* (2013) showed that water stress induced by high concentrations of PEG NaCl can significantly reduce callus growth parameters such as callus growth rate, relative growth rate, relative water content and percentage of living cells. However, calluses Under high osmotic pressure (NaCI and PEG) were able to adapt to the stress, created in the culture medium. The long-term culture of these calli, which seem to tolerate high osmotic. Pressures, allow them to further regenerate plants with the aim of selecting lines tolerant to water stress, and also to study the mechanism of cell tolerance to high osmotic pressures. Salinity and Drought are often interconnected, and plant responses to salt and water stress have much in common.

Ghasempour. II.R *et.a!* (2014) observed that Leaf, hypocotyls and roots explants which were cultured on MS medium with different concentrations of BAP, NAA, 2. 4-D, and KIN, as well as desolated combination of mentioned growth regulators were successfully propagated . The best result in terms of percentage response (97.79%) and nature of the callus induction were obtained with the combination of BAP 11 mg 1-1) in case of leaf-explants after 25-30 days. Also, about 96.97% of the hypocotyls of explants successfully produced callus with the combination of 2,4-D (0.5mg I-1), NAA (0.5mg I-1) and BAP (0.5 mg 1-1) as above after 25-30 days. The percentage response in case of root explants was observed quit low in comparison to the leaf and'

shoot tip i explants.

Significance of the study

In vitro selection has emerged as a feasible and cost-effective tool for developing salt and drought tolerant plants. Plants tolerant to both the biotic and the abiotic stresses can be acquired by applying the selecting agents such as NaCI (for salt tolerance), PEG or mannitol (for drought tolerance) in the culture media. Only the explants capable of sustaining such environments survive in the long run and are selected...*In vitro* selection is based on the induction. of genetic .variation among cells tissues and organs in cultured and regenerated plants. The selection of somadonal variations appearing in the regenerated plants may be genetically stable and useful in crop improvement.

Considering the potential biotechnological applications of issue culture present study is of great significant to develop salt and through tolerance genotype in safflower.

6. PROPOSED WORK PLAN/ FORMULATION AND STRUCTURE OF STUDY

Period of study	Achievable targets
First Year	1) Collection review, compilation of synopsis and submission 2) Standardization of *in-vitro* regeneration protocol for elite safflower genotypes
Second Year	1) *In vitro* screening of calli for salt tolerance 2) Physico-Biochemical studies of stressed tolerant calli
|Third Year	Compilation of data, analysis of data, submission of final synopsis and thesis

7. REFERENCES

Ashrat M.Harris P.J.C. 2004 potential biochemical indicators of salinity tolerance in plants. Plant Sci. 166,3-16

Bajji M., Bertin P.Lutts S.Kinet JM2004 Evaluation of drought resistance? related traits in durum wheat somaclonal lines selected in vitro Alst J.Exp. Agric 44, 27-55.

Dics M.A. Rispal N, Prats, E. Rubiales D. Singh, K.B., 2006 Biotechnology Approches to overcome Euphyt-ica 147,1-24

Eaishadfaei Elyasi P. Aghaee M. 2012 in Vitro Selection for Drought Tolerance in Common Wheat (Triticum aestivum L.) Genotypes by Mature Emboryo Culture Anerican.1 of Sci. Res. 48,102-115.

Gawande N.D., Mahurkar D.G., Rathod T.U., Jahagidar S.W., Shinde M. 2005. In vitro Screaming of wheat genotype for drough rolerance Annals plant Physiol, 19,162-168

Ghasempour H.R. Zh. Sohelikhan, A.R. Zeharjadi, S. Chasenpour and N. Karini, 2014 invitro Micro Propagation, callus induction and shoot regeneration in safflower L.cv. Lesaf3 International of plant physiology 4 (2) 999-1004.

Hassion D., Bouharmont J. 1994 In vitro selection and characterization of drought toleant plants of wheat (Triticum durum desf) Agronomy 14, 65-70

Hassion N.M., Serag, M.S. B-Feky, F.M. 2004 changes in nitrogen content and pro- following invitro selection of NaCl resistant nungheanandonato Acta physiol. Plant 26,

Iemg Z.H.w and Y.H. Wang, 2005, Separation and determination of chalcones from Carthamum tinctorius L. and is medicinal preparation by capillary zone electrophoresis I 28,1244-1247

M.M.Mansouri, M.R.Abdollahi and F. Moradi(2013) Effect of NaCl and PEG Induced stress on callus growth paraneters of two safflower (carhamus tinctorius international journal of Agriculture and crop science, IJACS/20136-36-3127-13

Anowls 1989 Safflower In oil crops of the world in downey B.K. rohella C Ashri A (d) New York, p 363-374

Lupi, Bennic F. Lowi and D. Cenai 2004, plantlet formation from callus and shoot tip culture inducd sonachnad variation of Wheat for Dhought Taolance. I Agric 50 (2)

Mahmood Razzaq A. Ashraf M. Haiz HA, Kaleen Sh. Qayyan A., Ahmad M. 2012 In vitro issue culture induced sonadonal variation of wheat for Drought Toleance J. Agic (2)

Musdal AKA Gupta S.D. (2001) Direct shoot organogenesis and plant regeneration in soffower In vitor Cellular and Dev. Biol. 37(1) 50-54.

Munns 2002 comparative physiology of salt and water strees, plant cell Environ 25,239-250.

Narshimha Rao N et.al (2008) Establishment of regeneration and transformation protoeds in safflower 7th intenation conference Australia.

Shabhani A., Sepaskha A.R. Kangar Haghighi A.A. 2013 Growth and physiologic response of deficit irrigation water salimty and planning method. liner 1 of plant 69-396

Sujatha N.E., Dinesh K.V. (2017) In vitro bud regeneration of carthamus tinctorius and carthamus Species from leaf explants and axilary huds Biologia Plantarum volume 782-786.

Wang B., Vinocaur B., Altman A., 2003 Plant responses to drought salinity and excrene temperatures genetic engineering for stress tolerance planta 2018,1-14

Yang ME Bengman JW(1992) Agoducterim tunefleiens mediated transformation of safflower centernial plant cell Rep. 11(111) 581-585.

Zair, Sabonunji K., Titalsen M. Chiyah H.2003 Salt tolerance improvement in some Cultivar after application of in vitro selection pressure plant cell tises Organ Cult 73, 237-291

Zurich Saneilikhah (2013) Effects of saline and mamitrol induced stress on some biochemical and physiological parameters of carthanus tinctorius L varieties callus cultures AICS 1874

Research Scholer

(Wagh H.A.)
Research Centre in Biotechnology
Swami Vivekanand Mahavidyalaya , Udgir.

Research Guide

(Dr. B. M. Kareppa)
(M.Sc., Ph.D., F.B.S.)
Research Guide & Head,
P.G.Dept. of Biotechnology
D.S.M. College, Parbhani.